Chlamydomonas reinhardtii Fea1 Protein Facilitates Iron Uptake in Transgenic Plants

A Senior Honors Thesis

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By

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Introduction

As the leading human nutritional disorder in the world today, iron deficiency affects about 43% of women and 34% of men worldwide (Stephenson et al., 2000). The functional effects of iron deficiency anemia result from a reduction in the circulating hemoglobin and myoglobin, as well as in a reduction in iron-containing enzymes. The major consequences are reduced psychomotor and mental development in infants (Walter et al., 1986), poor pregnancy outcome (Murphy et al., 1986), decreased immune function (Murakawa et al., 1987), tiredness, and poor work performance (Basta et al., 1979). Blood losses due to heavy menstrual cycle and worm infections are some of the common ways that iron deficiency anemia can be aggravated. Unfortunately, as a direct consequence of malnutrition and various disease and infections, 90% of the world's anemic population lives in the third world countries concentrated mainly in Southeast Asia and Africa (Stephenson et al., 2000). Currently, many researchers worldwide are devoting their time and efforts to reduce the number of iron deficiency-related disorders by engineering crops for increased iron content. Many iron-uptake or iron-regulation genes from plants have been cloned and are being analyzed for their role in increased iron accumulation in crop plants (Curie and Briat, 2003).

In 2002, one such gene, *FEA1*, was discovered from the green alga *Chlamydomonas reinhardtii* (Rubinelli et al., 2002). Following exposure to toxic levels of cadmium or reduced levels of iron, expression of *FEA1* transcript levels increased dramatically (>50X). Furthermore, the *FEA1* gene was able to rescue the characteristic slow-growth phenotype of *Saccharomyces cerevisiae fet3fet4* mutant strain that is impaired in iron uptake and transport. These results indicated that the Fea1 protein might

be a candidate for an iron assimilation component (Rubinelli et al., 2002) in an as yet unknown pathway (Figure 1). Additional evidence supporting a role for Fea1 in iron uptake was the observation that a homolog (Hcr1) of Fea1 protein was secreted by *Chlorococcum littorale* when cultured in high CO_2 or low iron suggesting a role for Hcr1 in iron assimilation in this green alga as well (Sasaki et al., 1998). Furthermore, expression of Fea1 protein resulted in a 2-fold increase of iron-assimilation per yeast cell (Rubinelli et al., 2002). Interestingly, to date, no homologues of *FEA1* have been identified in any organisms other than eukaryotic algae.

Since iron is not readily available to plants due to its low solubility (Robinson et al., 1999), and since plants serve as the primary source of dietary iron for most of the world's population, we have expressed *FEA1* gene in *Arabidopsis thaliana* to determine whether expression of this gene could enhance iron uptake in plants. To test this hypothesis, the *FEA1* gene was expressed constitutively under the control of 2X35S CaMV promoter and root-specifically under the control of patatin promoter from potato. We present here data that show that the *FEA1* rescues the lethal *IRT1* iron transporter mutant of Arabidopsis. Furthermore under iron limiting growth conditions, transgenic plants expressing the FEA1 gene were able to perform better than wild-type plants in growth and iron stress response, suggesting an enhanced accumulation of iron by Fea1. Future experiments on elemental analysis should reveal actual iron concentrations and help to determine Fea1's ability to increase iron content in plants. Introducing the *FEA1* gene to global crops such as cassava might be an effective means to eliminate iron deficiency.

Materials and Methods

Plant Growth Conditions

Wild-type seeds of *Arabidopsis thaliana* (ecotype Columbia) were surface-sterilized, placed in the dark at 4°C for 2 days, and then sown on plates of Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2% sucrose, 1 mM Mes, and 0.7% agar, pH 5.7 with KOH. Transgenic plants were selected on plates supplemented with kanamycin (40 μg/mL) and vancomycin (500 μg/mL). Plates were incubated at 23°C under constant illumination for 10 to 14 days until they reached the four-to six-true-leaf stage. Seedlings were then transferred to Metro-mix or Fafard #2 potting soil (Conrad Fafard, Inc., Agawam, MA) after selection. All seeds had reached the fourth generation (T4) upon experimentation so as to generate homozygous transgenics.

T4 seedlings were transferred to either iron sufficient [50 μM Fe(III)-EDTA] or iron deficient {0 μM Fe(III)-EDTA or 300 μM FerroZine [3- (2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate]; Sigma-Aldrich, St. Louis, MO}plates. The medium contained 1% sucrose, macronutrients: 5 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, micronutrients: 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM Na_2MoO_4, 10 mM NaCl, 0.01 mM $CoCl_2$, and 0.8% agar, pH 5.8. Plants were incubated for various times after transfer in a growth chamber as described.

Plasmid Construction and Plant Transformation

For transformation of wild-type plants, the *Chlamydomonas reinhardtii FEA1* cDNA was cloned as *Xho*I and *Sst*I fragments behind 2X35S (enhanced *Cauliflower mosaic virus*) or patatin (potato, Solanum tuberosum L.) promoter. The vector backbone

was pKYLX (Invitrogen). Using standard methods the recombinant vectors were transformed into DH5alpha E.coli cells and sequence analyzed at the Plant Microbe and Genomics Facility at The Ohio State University. The 2x35S:*FEA1* and Pat:*FEA1* construct were used to transform *Agrobacterium tumefaciens* strain LBA4404 by electroporation and selected in medium containing tetracycline (30 µg/mL) and streptomycin (50 µg/mL). Agrobacterium-mediated transformation of wild-type Arabidopsis plants (Columbia) was accomplished using floral-dip method (Clough and Bent, 1998). T1 seeds obtained from self-fertilization of the primary transformants were surface-sterilized and grown on MS medium supplemented with kanamycin (40 µg/mL) and vancomycin (500 µg/mL). The antibiotic resistant plants were transferred to soil and self-fertilized to obtain the T2 seeds. The T2 seeds were surface-sterilized and screened for kanamycin resistance. This procedure was repeated to obtain T4 seeds.

For transformation of the Arabidopsis *IRT1* mutant (Arabidopsis Biological Resource Center, Columbus, OH), the *FEA1* cDNA was cloned as *Sma*I and *Sst*I fragments behind patatin promoter using vector pBI121 that carried the kanamycin resistant gene.

DNA and RNA Analysis of Transgenic Plants

Leaf genomic DNA was extracted and the *FEA1* insert was PCR amplified using *FEA1*F1 (5'-CAAGCCCGTCGCACAGTTAAC-3') and *FEA1*R1 (5'-GCCTTGAAGTTGCGCAGCTTG-3') primers (final concentration of 0.4 µM) according to the manufacturer's instructions (REDExtract-N-Amp Plant PCR Kits, Sigma-Aldrich, St. Louis, MO).

Total RNA was extracted (Rneasy Plant Mini Kits, QIAGEN, Valencia, CA) from root, stem, and leaf tissues of plants cultivated as described above. Reverse transcriptase-mediated PCR (RT-PCR) experiments were performed on total RNA treated previously with RNase-free DNase. The primers used for *FEA1* detection were *FEA1*F1 and *FEA1*R1, amplifying an 850 bp fragment. The control used in RT-PCR was actin; a 400 bp product was amplified from the actin mRNA.

Cotyledon Emergence

Forty seeds from wild-type and transgenic plants were surfaced-sterilized and sown on plates that were either iron sufficient [50 μM Fe(III)-EDTA] or iron deficient [0 μM Fe(III)-EDTA]. After 2 days of darkness at 4°C, cotyledon emergence was carefully observed at 23°C under constant illumination for 10 days. The number of seeds showing healthy and complete cotyledon emergence was recorded everyday beginning at day 1. Each value is the mean of three experiments.

Root Growth Analysis

Seeds from wild-type and transgenic plants were surfaced-sterilized, placed in the dark at 4°C for 2 days, and sown on MS medium described. After 9 days of incubation at 23°C under constant illumination, plants were transferred to large plates that were either iron sufficient [50 μM Fe(III)-EDTA] or iron deficient [0 μM Fe(III)-EDTA] in a manner that allowed their roots to extend in a straight a line across the surface of the agar. Plates were placed in the growth chamber vertically so that the roots grew down along the surface of the agar. Root pictures were taken at day 10.

pH Assay

The pattern of pH change around wild-type and transgenic roots was visualized by placing seedlings on medium containing 0.2 mM $CaSO_4$ and the pH indicator Bromocresol Purple (0.006%), solidified with 0.7% agar. The pH of the medium was adjusted to 6.0 with NaOH. The plants had been germinated and grown to the four-to six-true-leaf stage on MS medium, and transferred to either iron sufficient [50 μM Fe(III)-EDTA] or iron deficient {300 μM FerroZine [3- (2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate]} plates for 3 days before the pH assay was performed.

Chlorophyll Fluorescence Analysis

Wild-type, IRT1 and transgenic seeds were surfaced-sterilized and sown on plates that were either iron sufficient [50 μM Fe(III)-EDTA] or iron deficient [0 μM Fe(III)-EDTA]. After 2 weeks, seedlings were subjected to 2 sec of actinic light (sensitivity 70%, irradiance 30%, electronic shutter at 500^{-1}s or 30000^{-1}s) and the chlorophyll fluorescence intensity was measured every 0.04 sec for 5 sec using a kinetic fluorescence CCD camera. Each curve represents a general pattern shared by multiple areas on several seedlings of the same plant.

Results

To investigate the role of Fea1 in iron assimilation in plants, the Arabidopsis iron-uptake mutant line *IRT1* was transformed with the *FEA1* gene from *C. reinhardtii* using the floral dip method (Clough and Bent, 1998). The *FEA1* cDNA was cloned as *Sma*I/ *Sst*I fragment behind the root-specific, patatin promoter (Figure 2B). Transformation of *A. tumefaciens* strain LBA4404 was performed by electroporation using the pBI121 vector that carried the modified T-DNA region. PCR based amplification of genomic DNA confirmed the presence of *Fea1* as an 850bp fragment using the *FEA1*F1 and *FEA1*R1 primers (Figure 3). Characterization of *IRT1* mutant showed that IRT1 is essential for the uptake of iron from the soil. *IRT1* knockouts display chlorosis and have a severe growth defect that eventually leads to death (Vert et al., 2002). Here we showed that *FEA1* was able to rescue the lethal phenotype of the *IRT1* mutant. At the four-to six-true-leaf stage, *FEA1* complemented IRT1 plants showed normal chlorophyll content and seedling size compared to *IRT1* mutant that suffered from severe chlorosis and stunted growth (Figure 4A). After 5 days in soil, the *FEA1* complemented IRT1 plants grew normally while the *IRT1* mutant had no significant change in size and chlorosis persisted (Figure 4A). In 5 weeks, the *FEA1* complemented IRT1 plants had produced flowers and siliques while the *IRT1I* mutant failed to develop beyond the four-to six-true leaf stage. Only a short yellow floral stalk producing one flower with chlorotic sepals developed on the untransformed mutant plants (Figure 4B). Fertility was recovered by *FEA1* complementation as T4 transgenic plants were shown in the growth study (Figures 4A and 4B).

Another Arabidopsis mutant, *pgr1*, carrying a single amino acid alteration in the Rieske subunit of the cyt b_6f complex has shown a conditionally impaired Q-cycle activity (Okegawa et al., 2005). Since iron is an essential component of the cyt b_6f complex, we hypothesized that *FEA1* could complement any functional loss of the cyt b_6f complex as a result of iron deficiency. Seeds from *FEA1* complemented IRT1 plants and the *IRT1* mutant were grown on iron sufficient medium for 2 weeks and subjected to 2 sec of light following dark adaptation to monitor chlorophyll fluorescence induction kinetics. Fluorescence intensity of the *FEA1* complemented IRT1 plant increased to about 1600 relative units 0.5 sec after the onset of illumination and dropped significantly to about 1000 relative units 5 sec after the onset of illumination, exhibiting the normal Kautsky curve. Fluorescence intensity of the *IRT1* mutant rose above 1700 and only decreased slightly to about 1400 at 5 sec. The decrease in chlorophyll fluorescence intensity in *FEA1* complemented IRT1 plant was twice that of *IRT1* mutant (Figure 5). Normal patterns of chlorophyll fluorescence activity were observed under iron sufficient condition for wild-type plants. The *IRT1* mutants could not germinate or grow large enough for chlorophyll fluorescence measurements under iron deficient condition.

To further examine Fea1's ability to uptake iron in a model plant, Arabidopsis (ecotype Columbia) was transformed with the *FEA1* gene using the floral dip method (Clough and Bent, 1998). The *FEA1* cDNA was cloned as *Xho*I and *Sst*I fragments carrying either a patatin or 2X35S promoter (Figure 2A). The pKYLX vector carrying the modified T-DNA region was transformed into *A. tumefaciens* strain LBA4404 by electroporation. RT-PCR experiments confirmed the expression of the *Fea1* gene in the leaf, stem, and root tissues of transgenic plants. The resulting 850bp fragments were

obtained using the *FEA1*F1 and *FEA1*R1 primers with actin as a control (Figures 6A and 6B). When grown on iron sufficient medium for 2 weeks, *FEA1* transgenic plants carrying the 2X35S promoter showed a larger seedling size compared to wild-type (Figure 7). Transgenic plants carrying the patatin promoter also had the same characteristics and both types of transgenic showed this phenotype when grown under iron deficient [0 μM Fe(III)-EDTA] medium (data not shown).

Several experiments have shown that the germination of *nramp3 nramp4* double mutants, which fails to mobilize vacuolar Fe during seed germination, is arrested under low Fe nutrition and fully rescued by high Fe supply (Lanquar et al., 2005). We reasoned that *FEA1* transgenic plants might show early cotyledon emergence compared to wild-type when sown under iron deficient medium. Seeds from wild-type and transgenic *FEA1* plants carrying either promoter were sown on iron deficient [0 μM Fe(III)-EDTA] medium. Over 90% of transgenic seeds had full cotyledon emergence by day 5. However, cotyledon emergence was not observed on day 5 for wild-type seeds and it was below 80% on day 6. All plants had completed cotyledon emergence by day 8 (Figure 8A). A visual representation of the actual experiments was included (Figure 8B). Each curve was generated as a mean from three individual experiments. Similar results were obtained from plants that were grown on iron sufficient medium (Figure 9).

Since root hair growth is enhanced under iron deficient conditions (Vert et al., 2002), we investigated root growth of wild-type and *FEA1* transgenic plants under iron replete and deficient growth to determine whether FEA1 expression reduced the need for additional root hair growth under iron deficient conditions in wild-type transgenic plants. After 9 days of growth on MS medium, wild-type and *FEA1* transgenic plants carrying

the patatin promoter were placed on either iron sufficient [50 μM Fe(III)-EDTA] or iron deficient [0 μM Fe(III)-EDTA] medium vertically along the surface of the agar for 10 days. Wild-type plants grown under low Fe conditions developed more lateral root branches and root hairs compared to transgenic plants at the same level on the agar surface (Figure 10A). At the root tip, extensive root hair formation was observed for wild-type while transgenic plants had no or very little root hairs (Figure 10B). Lateral root elongation is induced by low nitrate and/or phosphate availability (Linkohr et al., 2002) and extra root hairs result from limiting iron availability (Schmidt et al., 2000), both measures serve to increase surface area for more efficient nutrient absorption. These responses were fully elaborated by iron deficient wild-type plants whereas *FEA1* transgenic plants were not affected (Figures 10A and 10B) under iron deficient growth conditions.

One major response of Strategy I plants to iron deficiency is acidification of the rhizosphere (Yi et al., 1994). To determine whether *FEA1* transgenic plants showed this response, a medium acidification assay was performed. Plants that had been grown for 3 days on iron sufficient [50 μM Fe(III)-EDTA] or iron deficient {300 μM FerroZine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate]}medium were transferred to agar plates containing the pH indicator Bromocresol purple. Roots of iron deficient wild-type plants reduced the pH of the medium to below 5.2, as indicated by the changed of the indicator from red (at pH 6.0) to yellow. On the contrary, iron sufficient plants and growth of transgenic plants on iron deficient media did not result in acidification of the medium (Figure 11).

In addition to measuring chlorophyll fluorescence under iron sufficient condition, wild-type, *FEA1* complemented IRT1 plants, and *FEA1* transgenic plants carrying the patatin promoter were subjected to 2 sec of light after 2 weeks of growth on iron deficient [0 μM Fe(III)-EDTA] medium. Wild-type plants exhibited a dramatic increase in fluorescence intensity, reaching above 500 relative units at 0.5 sec and flattened afterward. There was no significant decrease in fluorescence intensity from 0.5 to 5 sec. The *FEA1* transgenic plant had chlorophyll fluorescence intensity just above 400 relative units at 0.5 sec and dropped to about 250 by 5 sec, demonstrating the usual Kautsky curve. For the *FEA1* complemented *IRT1* plant, fluorescence intensity increased to slightly above 350 at 0.5 sec and dropped to about 275 at 5 sec. Although the decrease in intensity was not dramatic, it did not reach a plateau as demonstrated by wild-type. The decrease in fluorescence intensity for *FEA1* transgenic plant was 3-fold greater than wild-type (Figure 12).

Discussion

The data presented here offer insight into the function of Fea1 in dicotyledonous plants. Although the pathway responsible for iron uptake by Fea1 is still unclear, this work provides strong evidence that Fea1 is an iron transporter. First of all, the lethal phenotype of *IRT1* mutant was fully rescued by complementation of *FEA1*. Chlorosis and growth defects were eliminated in *FEA1* complemented IRT1 plants (Figures 4A and 4B). Moreover, fertility was recovered and transgenic plants were able to produce normal flowers, siliques, and seeds when production of these structures has shown to be arrested under low Fe conditions (Waters et al., 2006). As a plasma membrane iron transporter that transports only Fe^{2+} (Curie and Briat, 2003), complementation of *IRT1* suggested that Fea1 transports Fe^{2+} (Rubinelli et al., 2002).

In addition to flower and seed production, iron is also essential in germination as iron deficiency halts germination in *nramp3 nramp4* double mutants (Lanquar et al., 2005). When compared to wild-type, *FEA1* transgenic plants showed rapid cotyledon emergence under both iron sufficient and deficient conditions, suggesting more iron storage in vacuoles and high iron uptake efficiency (Figures 8A, 8B and 9). Increased iron storage in vacuoles is also indicative of high iron uptake efficiency as T4 seeds were used in all experiments. Parent plants expressing Fea1 might be able to assimilate more iron and thus produce seeds that contained elevated levels of iron. During the first couple days of germination, iron supply comes solely from the vacuoles and *IRT1* expression begins to increase rapidly on the third day (Lanquar et al., 2005). Fea1 expression was able to increase initial iron supply from the vacuoles and accelerate iron uptake to generate rapid cotyledon emergence compared to wild-type plants when grown under

iron deficient medium. Such reasoning could also explain the large seedling size observed in transgenic plants since growth was enhanced by increased iron availability (Figure 7).

One typical response to iron deficiency in plants is to increase root surface area by root hair formation for maximum iron absorption (Schmidt et al., 2000). The dense and extensive root hair formation in wild-type roots grown under iron deficient [0 μM Fe(III)-EDTA] medium clearly demonstrated the iron stress response (Figures 10A and 10B). Increases in lateral root branching suggested either a lack of nitrate and/or phosphate (Linkohr et al., 2002) or a measure to produce more root hairs on the extra branches. Moreover, *IRT1* expression is shown to be localized in the root hairs and epidermis of iron deficient plants (Vert et al., 2002). Lateral root branching and root hair formation thus facilitated upregulation of *IRT1* to uptake more iron, a default iron stress response. The fact that *FEA1* transgenic plants showed normal lateral root growth and minimal or no root hair formation compared to wild-type (Figures 10A and 10B) suggests that Fea1 is a high affinity iron transporter and is able to supply adequate amount of iron even under iron deficient conditions.

Other than increasing lateral root branching and root hair formation under low iron conditions, Strategy I plants also acidify the rhizosphere through activation of a specific plasma membrane H^+-ATPase of the root epidermal cells to increase iron solubility (Curie and Briat, 2003). Iron deficient wild-type plants reduced the pH in the medium (yellow rhizosphere) whereas iron deficient *FEA1* transgenic plants raised the pH (purple rhizosphere) as if they were grown under iron sufficient conditions (Figure 11). The lack of iron stress response in transgenic plants after 3 days of iron deficient

{300 μM FerroZine [3- (2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate]} treatment showed that they were able to uptake iron more efficiently. When wild-type plants were exhibiting iron stress responses, transgenic plants were still able to utilize traces amount of iron from the medium due to the high affinity iron uptake ability of Fea1. Moreover, rapid iron uptake enhanced by Fea1 enabled transgenic plants to store more iron in less amount of time, thereby delaying the iron stress response. Also, roots of transgenic plants did not acidify the rhizosphere when iron in the medium ran out, regardless of initial iron treatment (data not shown). Therefore, transgenic plants were able to take up sufficient iron to avoid rhizosphere acidification.

Regulation of Q-cycle activity depends on a functional, iron-containing cyt b_6f complex, which mediates electron transport in photosynthesis and non-photochemical quenching (Okegawa et al., 2005). We have shown that the *FEA1* complemented IRT1 plant that was grown on iron sufficient medium showed lower chlorophyll fluorescence yield and a rapid decline in fluorescence after 2 sec of light exposure when compared to wild-type plants, indicative of higher photochemical efficiency and non-photochemical quenching by functional cyt b_6f complex (Figure 5). The same phenomenon was observed for *FEA1* complemented IRT1 and *FEA1* transgenic plants that were grown on iron deficient [0 μM Fe(III)-EDTA] medium (Figure 12). Under normal light conditions, photosynthesis occurs to make ATP for energy. When the reaction centers become saturated with excess light, the excitation energy must be dissipated by heat through non-photochemical quenching to avoid production of harmful triplet and singlet oxygen species (Dreuw et al., 2005). As a result, Fea1 enabled iron deficient plants to assimilate

more iron and build a functional cyt b_6f complex to mediate excess excitation energy, thus preventing photodamage.

The functions and characteristics of Fea1 in dicotyledonous plants presented in the paper serves as a model for genetic modification of crops in order to increase iron content in diet. Future experiments on elemental analysis of *FEA1* transgenic plants are needed to reveal the actual iron content. Further study of the protein should lead to a more detailed understanding of its iron uptake mechanism that in turn may contribute to engineering iron-rich crops, thus reducing the severity of iron deficiency worldwide.

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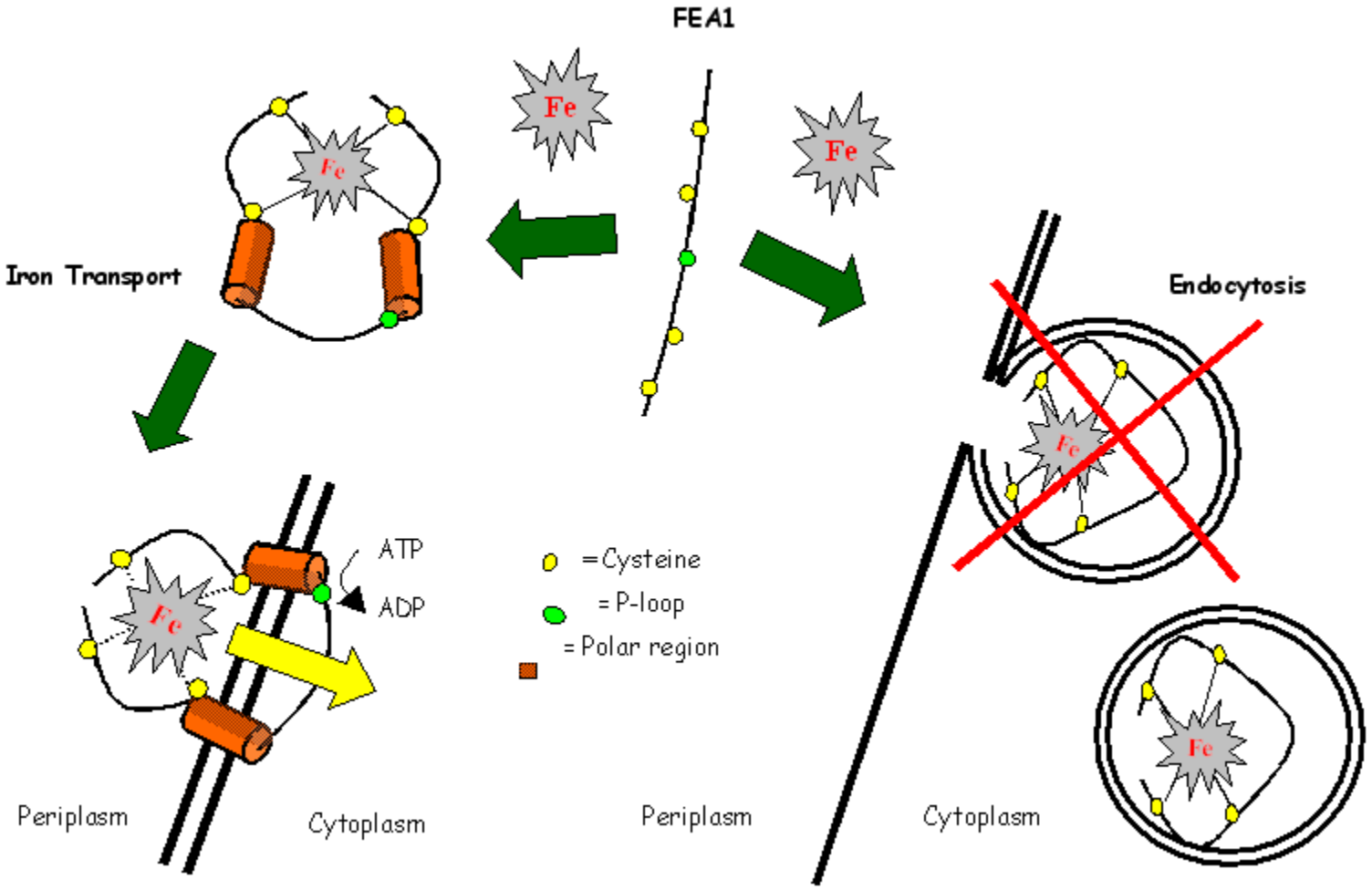
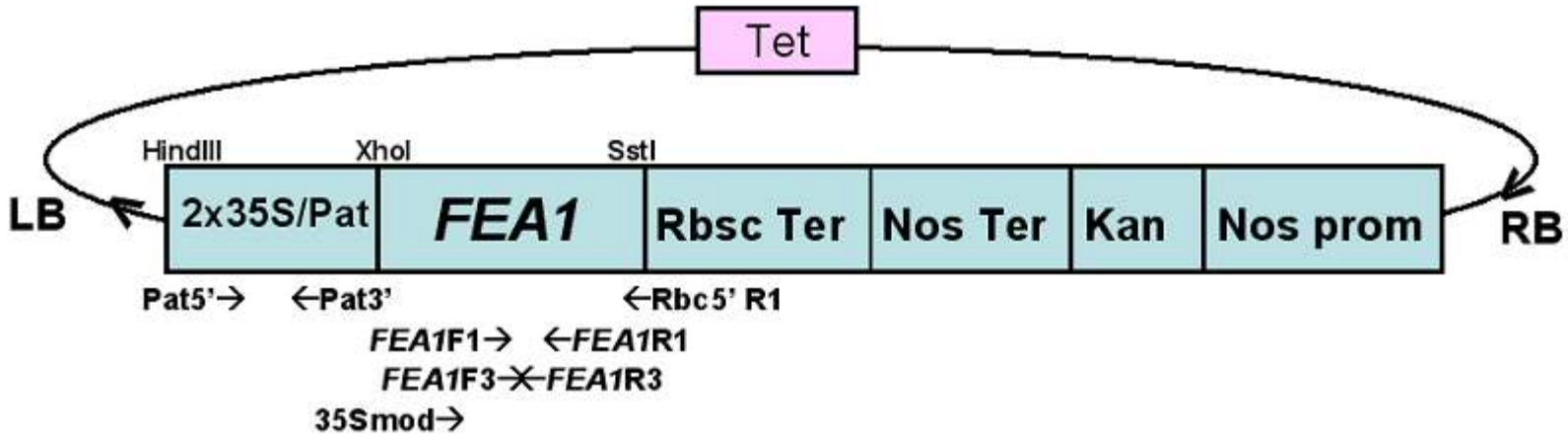


Figure 1: Schematic of the possible mechanisms for Fea1-mediated iron-uptake. The *FEA1* gene complements yeast Fe-uptake/endocytosis mutants thus favoring the iron transporter model. Fe binding to Fea1 presumably induces formation of a hairpin structure with two amphipathic helices that insert into the membrane forming a channel. Mutagenesis of the colored (yellow and green) residues blocks function.

(A)



(B)

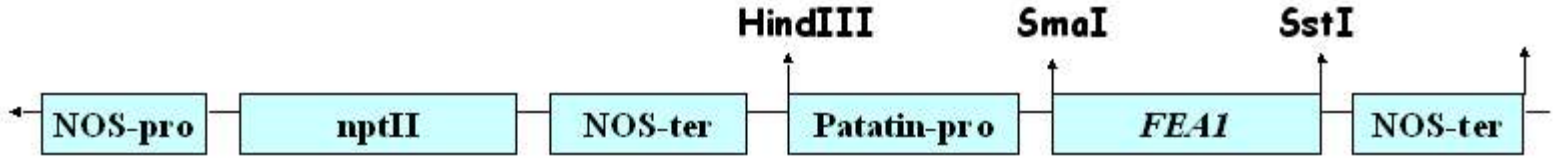


Figure 2: Constructs for transformation.
(A) Modified pKYLX vector containing either 2X35S or patatin promoter and *FEA1* for transformation of Arabidopsis. *Agrobacterium tumefaciens* (LBA 4404 strain) was used to transform the plasmid.
(B) The T-DNA region of the plasmid modified from pBI121 containing patatin promoter and *FEA1* gene used in the transformation of Arabidopsis iron-uptake mutant line *IRT1* for the complementation of iron-transport protein. The plasmid was transformed into *A. thaliana* with *Agrobacterium tumefaciens* (LBA 4404 strain).

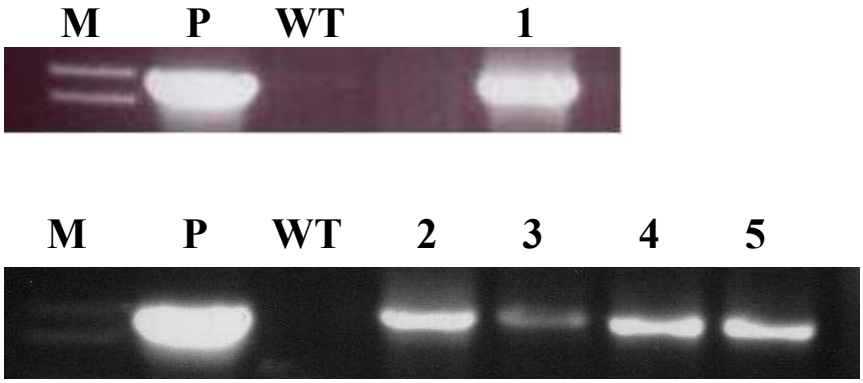


Figure 3: DNA analysis of *Fea1* complemented IRT1 plants. Leaf genomic DNA was extracted and the *FEA1* insert was PCR amplified using *FEA1*F1 and *FEA1*R1 primers, yielding an 850bp fragment. M: 1kb marker; P: plasmid control; 1: IRT1-FePat-A; 2: IRT1-FePat-C; 3: IRT1-FePat-D; 4: IRT1-FePat-E; 5: IRT1-FePat-F.

(A)

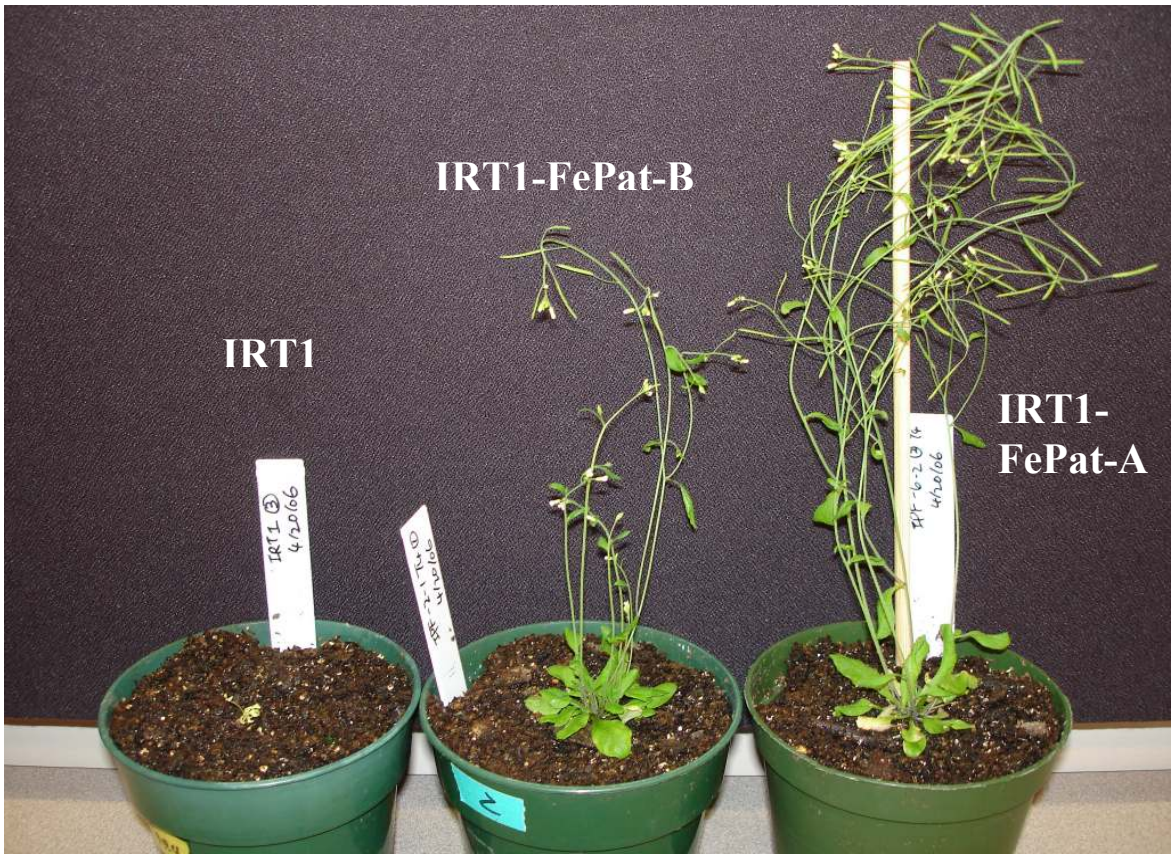
Day 1

IRT1-FePat-A **IRT1**

Day 6

Figure 4: General growth of *IRT1* mutant and *FEA1* complemented IRT1 plants. Seeds from *IRT1* mutant and *FEA1* complemented IRT1 plants were surfaced sterilized and sown on MS medium. At the four-to six-true-leaf stage, seedlings were transferred to soil. **(A)** Difference of growth between wild-type and *FEA1* complemented IRT1 plant in 5 days.
(B) Plants were grown on soil for five weeks.

(B)



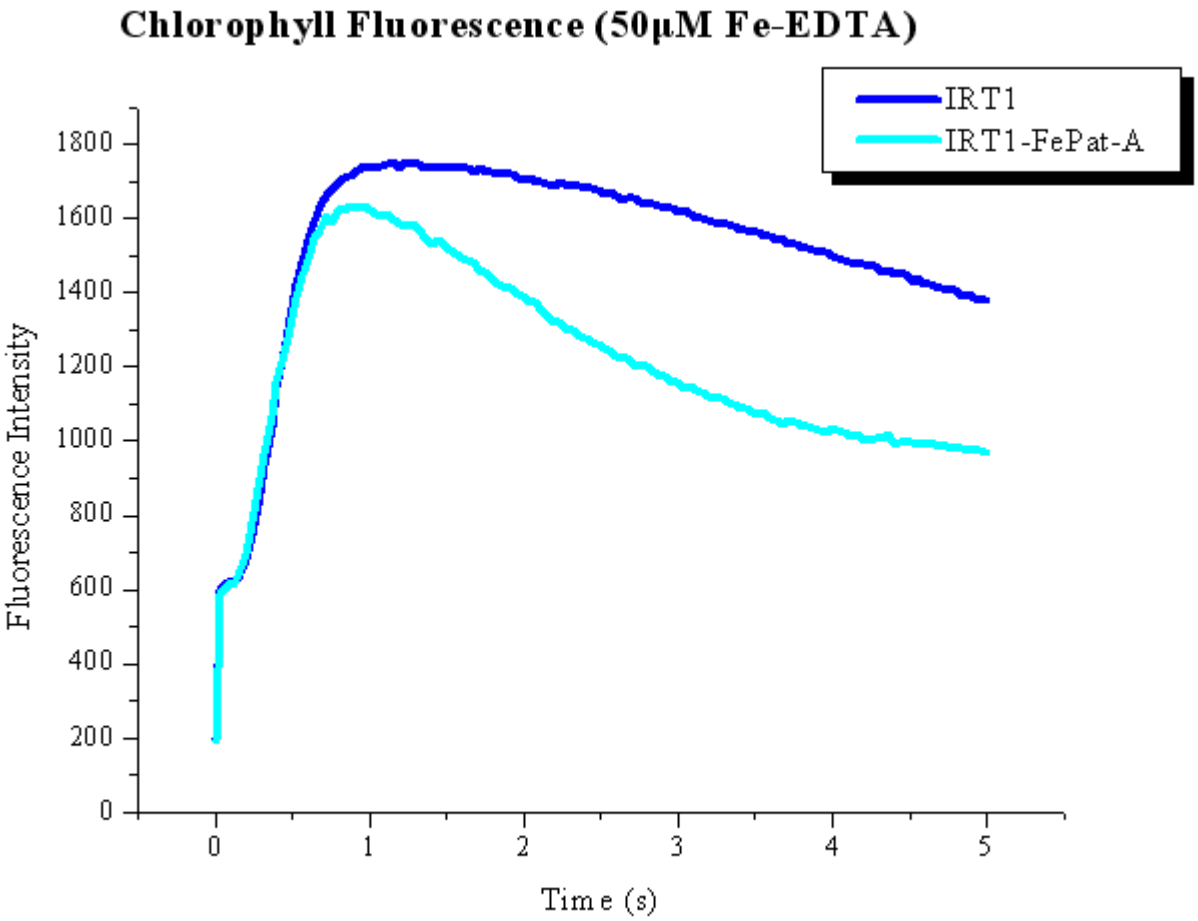


Figure 5: Chlorophyll fluorescence analysis of *IRT1* mutant and *FEA1* complemented IRT1 plant. Seeds from *IRT1* mutant and IRT1-FePat-A were surfaced sterilized and sown on plates that were iron sufficient [50 µM Fe(III)-EDTA] for 2 weeks. Fluorescence intensity was recorded by a kinetic fluorescence CCD camera every 0.04 sec for 5 sec after a 2 sec actinic light exposure.

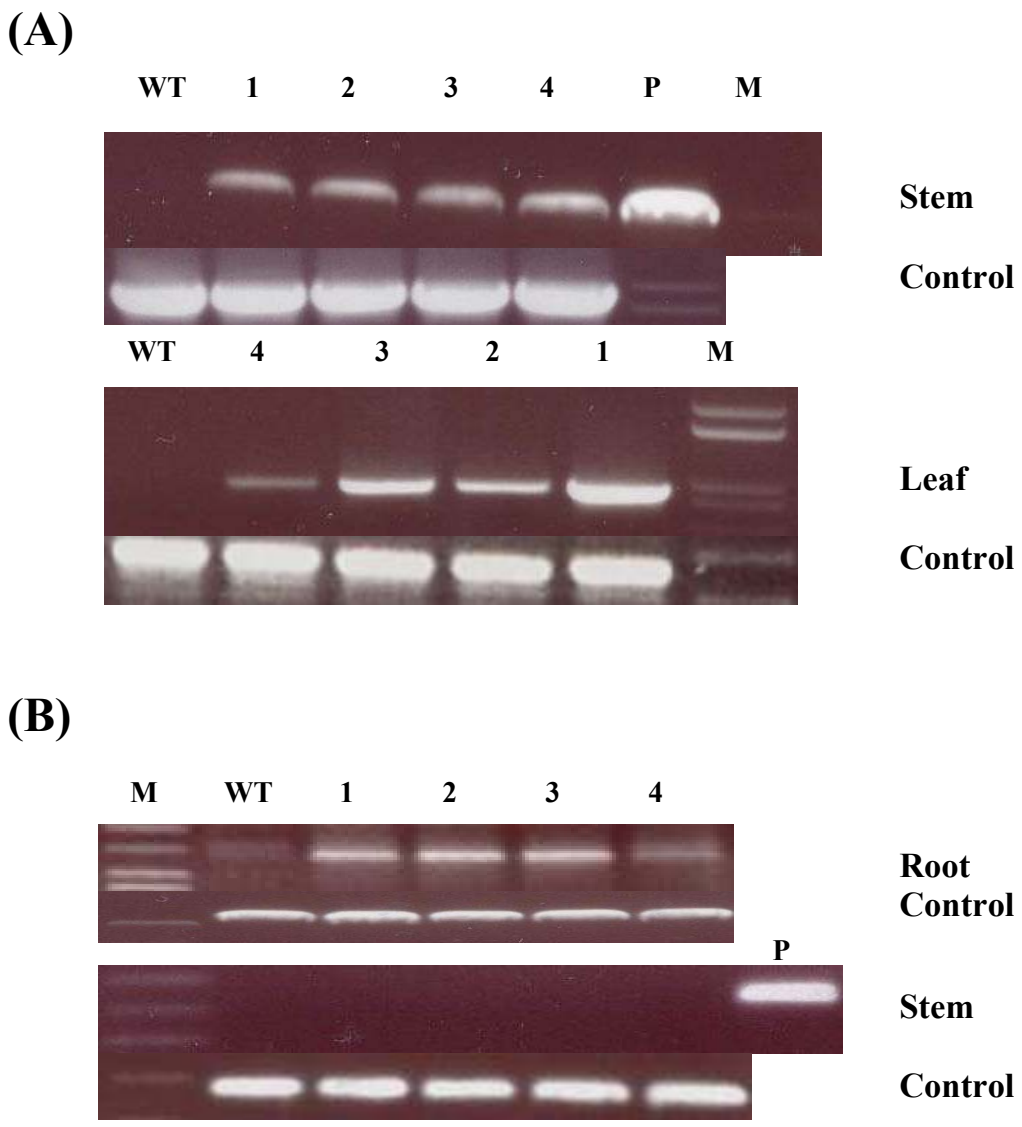


Figure 6: Tissue specific RNA analysis of *Fea1* transgenic plants. Total RNA was extracted from plants cultivated as described above. RT-PCR experiments were performed on total RNA treated previously with RNase-free DNase. The primers used for *FEA1* detection were *FEA1*F1 and *FEA1*R1, amplifying an 850bp fragment. Actin control was included as a 400bp fragment.
(A) Transgenic plants with 2X35S promoter. M: 1kb marker; P: plasmid control; 1: WT-Fe35S-A; 2: WT-Fe35S-B; 3: WT-Fe35S-C; 4: WT-Fe35S-E.
(B) Transgenic plants with patatin promoter. M: 1kb marker; P: plasmid control; 1: WT-FePat-C; 2: WT-FePat-D; 3: WT-FePat-F; 4: WT-FePat-G.

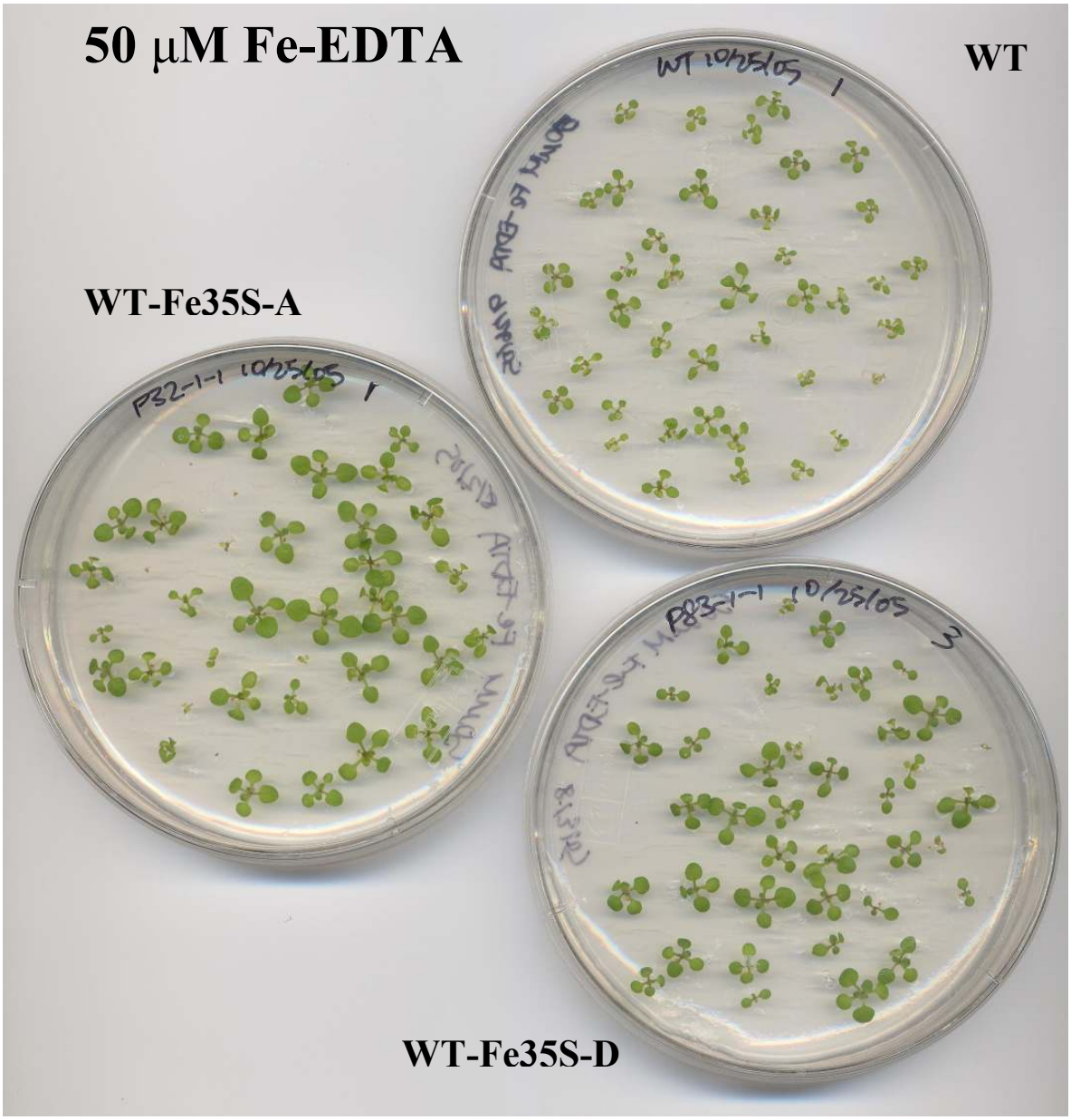
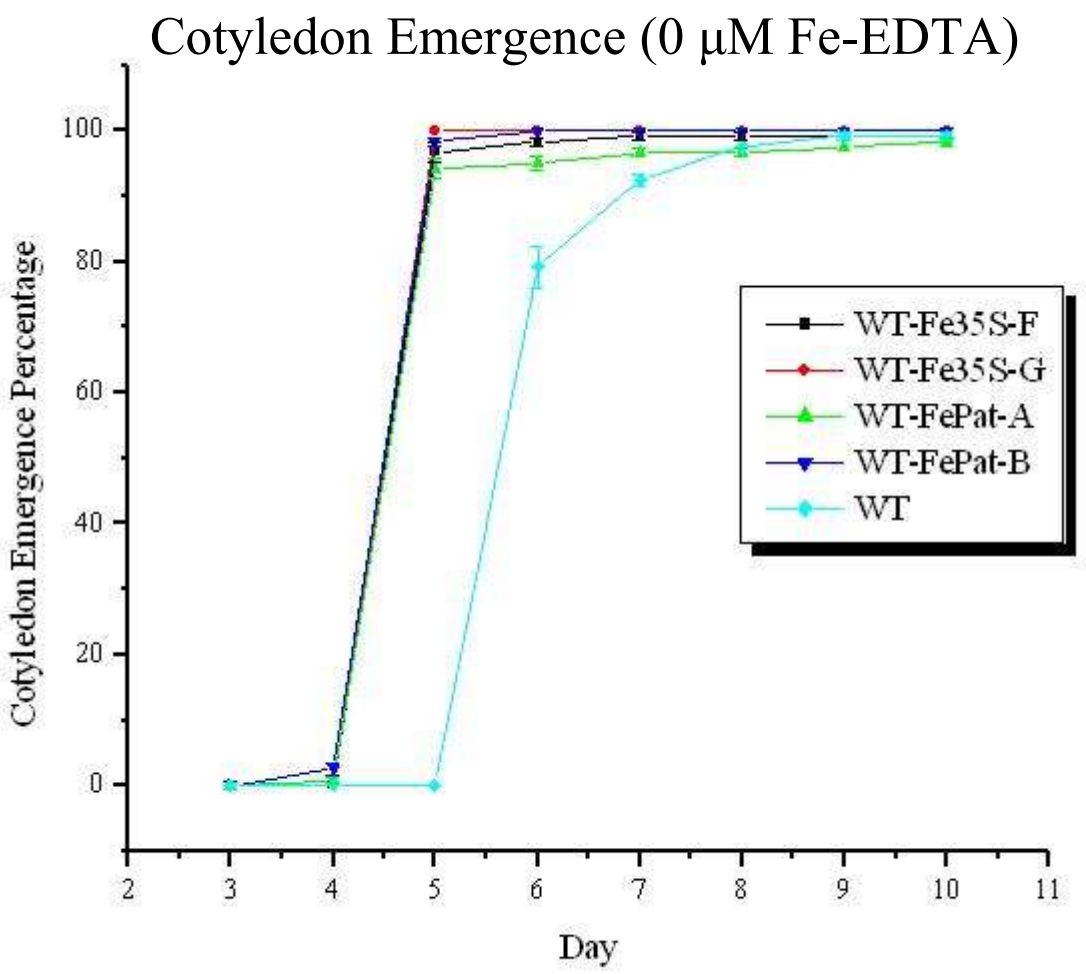


Figure 7: General growth of *FEA1* transgenic plants. Wild-type and transgenic seeds were surfaced sterilized and sown on plates that were iron sufficient [50 μM Fe(III)-EDTA]. Picture was taken after 2 weeks of growth.

(A)



(B)

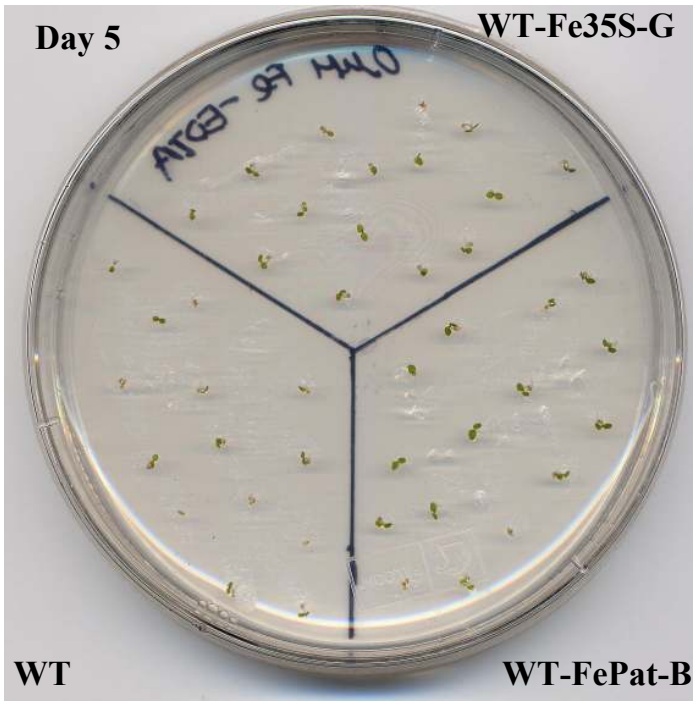


Figure 8: Cotyledon emergence of wild-type and *FEA1* transgenic seeds. **(A)** Wild-type and transgenic seeds were surfaced sterilized, placed in the dark at 4°C for 2 days, and sown on plates that were iron deficient [0 µM Fe(III)-EDTA]. Rate of cotyledon emergence was recorded daily beginning from day 1 through 10. Each curve represents the mean of three experiments.
(B) Picture showing different rates of cotyledon emergence at day 5.

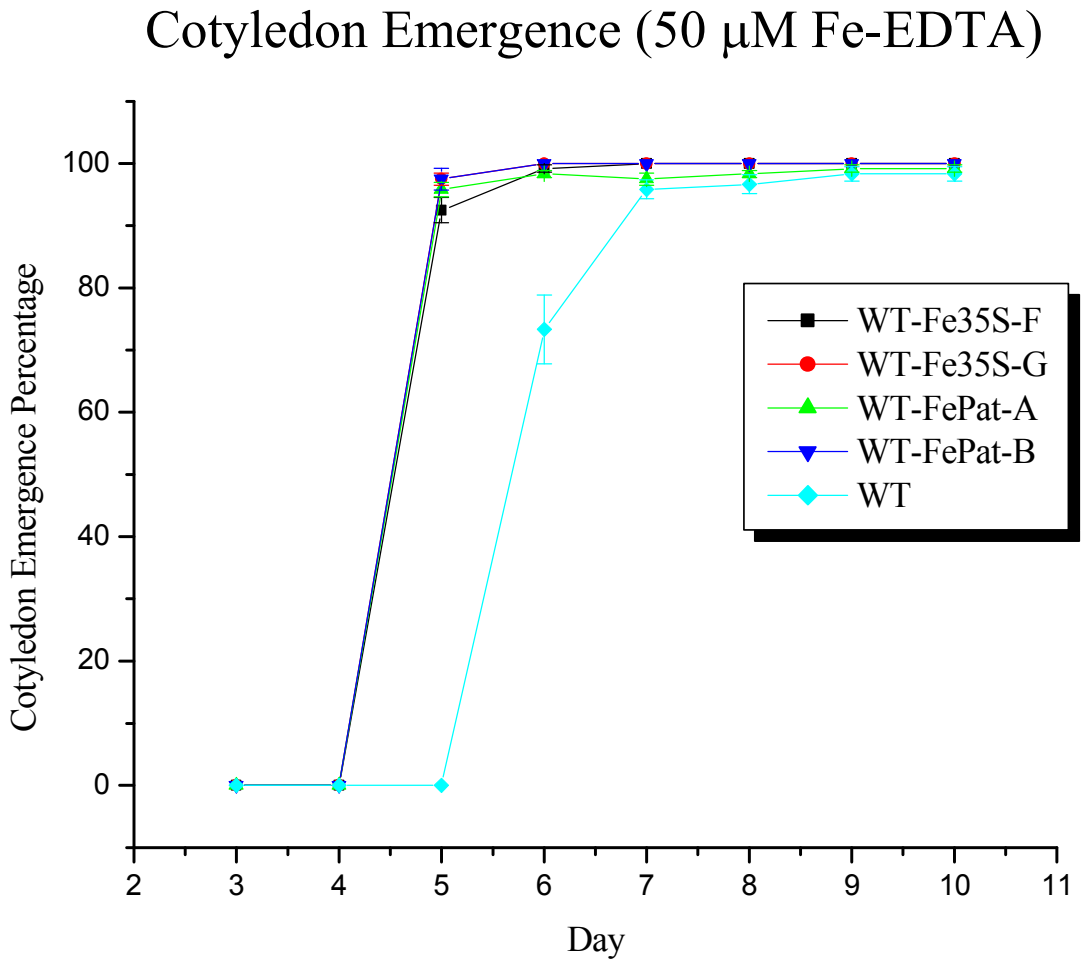
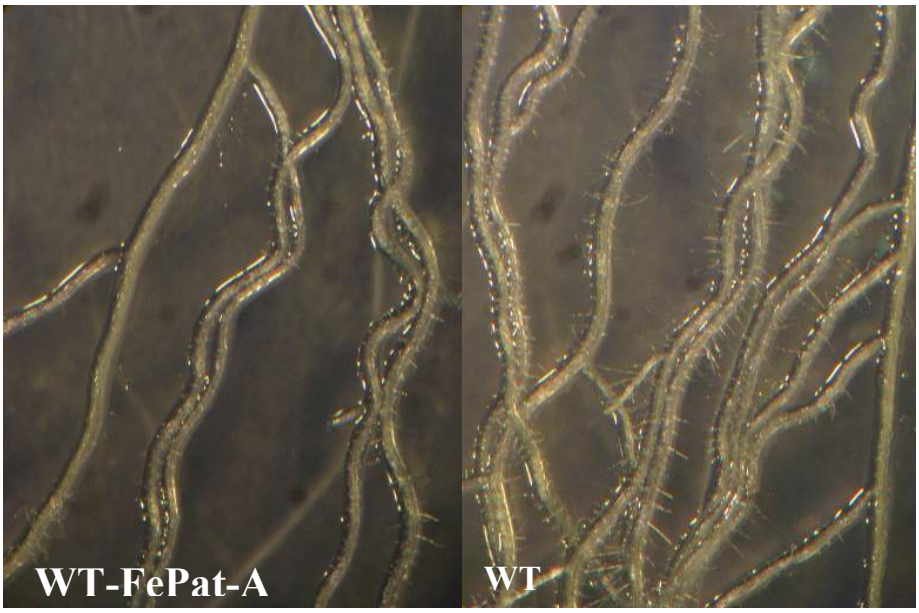


Figure 9: Cotyledon emergence of wild-type and *FEA1* transgenic seeds. Wild-type and transgenic seeds were surfaced sterilized, placed in the dark at 4°C for 2 days, and sown on plates that were iron deficient [50 µM Fe(III)-EDTA]. Rate of cotyledon emergence was recorded daily beginning from day 1 through 10. Each curve represents the mean of three experiments.

(A)



(B)

Figure 10: Root analysis of *FEA1* transgenic plants. Wild-type and transgenic seeds were grown on MS for 9 days and transferred to iron deficient [0 μM Fe(III)-EDTA] plates in a manner that their roots extended in as straight a line as possible across the surface of the agar. Plates were placed in the growth chamber vertically so that the roots grew down along the surface of the agar. Pictures were taken at day 10.
(A) Root branches immediately below the edge of the longest leaf. Pictures were taken at the same level on the surface of agar.
(B) Difference in root hair formation at the root tip.

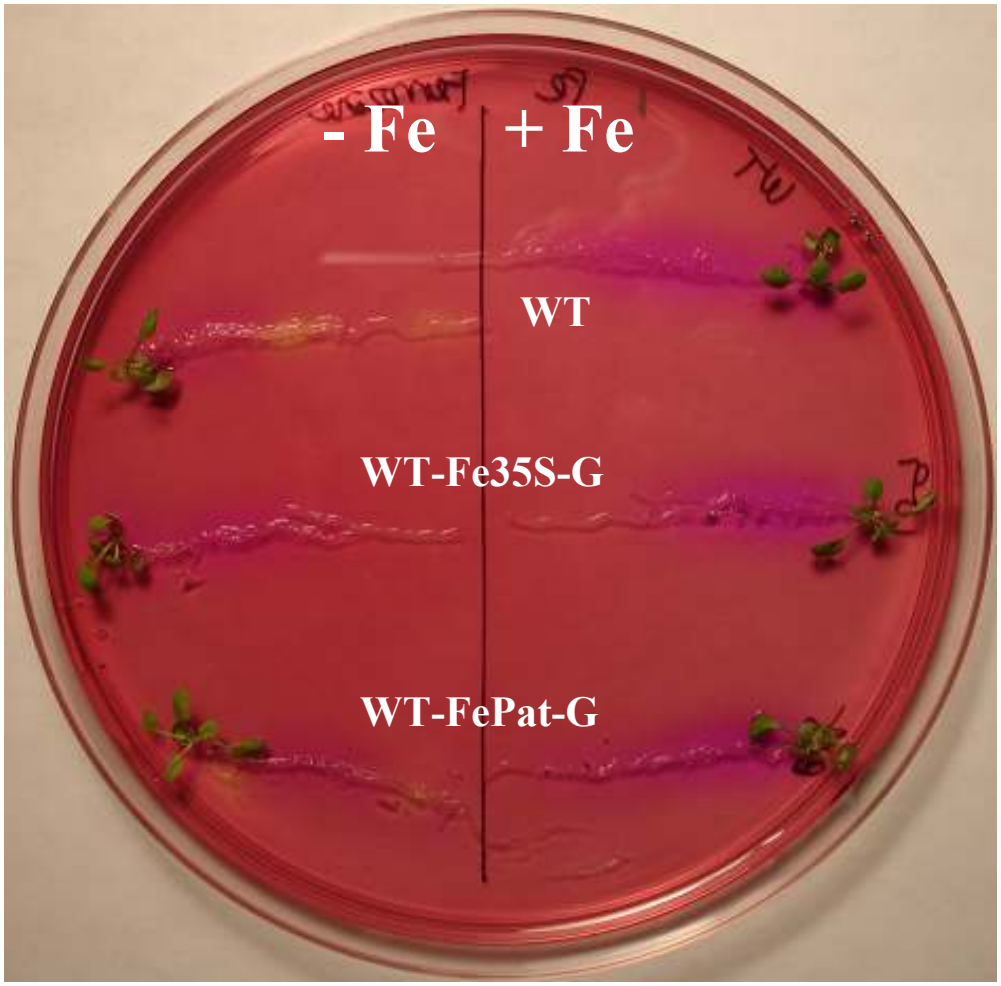


Figure 11: Acidification response of wild-type and *FEA1* transgenic plants. The plants were germinated on MS medium and transferred to either iron sufficient [50 µM Fe(III)-EDTA] or iron deficient {300 µM FerroZine [3- (2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate]} plates at the four-to six-true-leaf stage. After 3 days, plants were placed on a plate containing the pH indicator Bromocresol Purple. In 4.5 hours, roots of iron deficient wild-type reduced the pH of the medium to below 5.2, as indicated by the changed of the indicator from red (at pH 6.0) to yellow. Iron sufficient plants and iron deficient transgenics caused the pH of the medium to rise above 7.0, as indicated by the purple color around the roots.

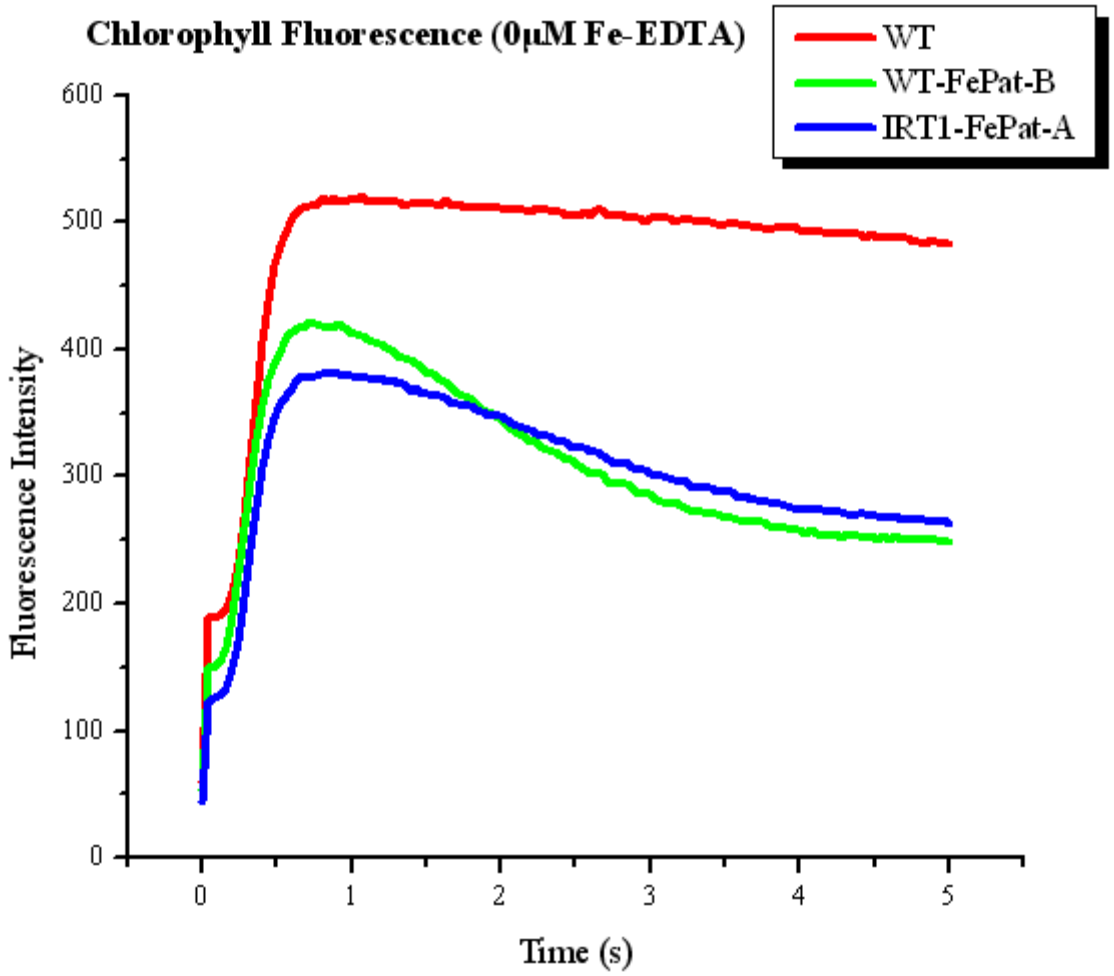


Figure 12: Chlorophyll fluorescence analysis of wild-type, *FEA1* complemented IRT1, and *FEA1* transgenic plants. Seeds were surfaced sterilized and sown on plates that were iron deficient [0 µM Fe(III)-EDTA] for 2 weeks. Fluorescence intensity was recorded by a kinetic fluorescence CCD camera every 0.04 sec for 5 sec after a 2 sec actinic light exposure.